CHAPTER 13.

FRACTIONATION OF FOOD BIOACTIVE OLIGOSACCHARIDES

Moreno F.J.[1], Carrero-Carralero C.[2], Hernández-Hernández O.[3], Sanz M.L.[2*]

[1] Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM). Madrid (Spain)

[2]Instituto de Química Orgánica General (CSIC). Madrid (Spain)

[3]Pure and Applied Biochemsitry. Lund University. Lund (Sweden)

* Corresponding author:

e-mail: mlsanz@iqog.csic.es

Telephone: + 34 91 5622900 (ext. 212)

Fax: + 34 91 5644853

Abstract

Bioactive oligosaccharides obtained either from natural or synthetic sources are normally present in highly complex matrices. This heterogeneity is particularly important at the carbohydrate fraction. In this context, the suitable fractionation of bioactive oligosaccharides becomes an important task to broaden their applications, as well as to accomplish their structural and/or functional characterization. This chapter deals with a wide number of techniques at semi- and preparative scale, i.e. membrane separation processes, chromatographic techniques, solvent-based methods, microbiological and enzymatic techniques, whose applications largely depend on the use and the purity required for the fractionated oligosaccharides. The advantages and major drawbacks of the different techniques are also discussed in detail. In addition to well-established techniques, such as ultrafiltration, nanofiltration, size-exclusion or ion exchange chromatography, this chapter will also describe novel and green techniques which have been recently reported to be useful for the prospective fractionation of oligosaccharides with high recoveries, including pressurized liquid extraction, supercritical fluid and ionic liquids.

Keywords: fractionation, membrane techniques, size exclusion chromatography, ion exchange chromatography, activated charcoal, pressurized liquid extraction, supercritical fluids, microbiological treatments, ionic liquids

13.1. Introduction

As indicated in previous chapters, bioactive carbohydrates, both naturally occurring and synthesized, are the result of mixtures with different degrees of polymerization (DP), glycosidic linkages or monosaccharide unit composition. Moreover, those present in natural products can be part of complex matrices constituted by other compounds including lipids, proteins, etc (Sanz & Martinez-Castro 2007).

Either for oligosaccharide production or analysis, fractionation and purification procedures are required. Regarding production of bioactive oligosaccharides, the removal of mono- and disaccharides fractions could be required to evaluate their functional properties (e.g. *in vitro* prebiotic activity), to obtain enriched bioactive fractions or to use them as food ingredients in specialized products for individuals with different disorders (diabetic people, lactose intolerants, etc.), as well as in low calorie foods with a reduction of some mono- and disaccharides.

Fractionation of oligosaccharides based on their glycosidic linkages or monosaccharide composition can also be crucial to the elucidation of carbohydrate structures, and consequently, to gain insight on the structure/function relationship. Although analytical techniques have noticeably advanced in the last years, chromatographic, spectrometric or spectroscopic tools are still not sufficient to achieve an exhaustive characterization of oligosaccharides present in complex mixtures. Only tentative identifications are carried out without a previous fractionation.

Among purification steps, centrifugation, precipitation and/or filtration are the most commonly used. These wide-ranging procedures are treated in each corresponding chapter and they will not be discussed here since this chapter will be focused on effective tools to fractionate specific bioactive oligosaccharides from complex

carbohydrate mixtures. Nevertheless, some exceptions will be made as it could be the case of the continuous enzymatic production of galactooligosaccharides (GOS) and their separation from the recycled enzyme or the fractionation of XOS from biomass both accomplished by membrane filtration processes (section 13.2).

Fractionation of oligosaccharides is not a straightforward task due to the structural complexity which also includes similarities in carbohydrate structures. The search of fractionation techniques to obtain both high selectivity and efficiency, spending low solvent volumes, environmentally friendly and inexpensive is one of the main aims of both researchers and industries.

This chapter deals with the different procedures used for the fractionation of bioactive carbohydrates for either producing functional ingredients or preparing samples for their further analysis. As it is summarized in **Figure 13.1**, this chapter will cover not only advances in traditional methods but also novel techniques with potential use in this field.

13.2. Membrane techniques

Membrane separation processes are normally driven by a pressure gradient in which the membrane fractionates components of a liquid mixture as a function of their solvated size and structure. Microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are the standard technologies employed for purification and/or fractionation of bioactive components (Akin *et al.* 2012), differing mainly in the membrane pore size and operating pressure. Parameters such as pressure, membrane type, temperature, sample pre-treatment, stirring, concentration and ionic environment influence the membrane filtration process. The industrial membrane configuration is

usually cross-flow filtration, which means that the solution to be filtered is flowing across the membrane surface at a determined velocity while the filtrate is going through the membrane. Nevertheless, other operational modes, such as dead-end membrane filtration, as well as free and immobilized enzymes in a membrane reactor (in the case of enzymatically produced oligosaccharides) can also be used for the fractionation of carbohydrate mixtures (Pinelo *et al.* 2009). Membrane-based techniques can be considered as the most feasible, technically and economically, down-stream strategy for industrial manufacture of enzymatically modified oligosaccharides (Pinelo *et al.* 2009). Among their advantages as compared to chromatographic purification techniques (section 13.3) are low energy requirements and operational complexity (Goulas *et al.* 2003).

Among the different membrane techniques, UF and NF processes are the most widely used to purify, concentrate and fractionate carbohydrates. Likewise, both types of membrane processes may also be used in series to improve the performance. **Table 13.1** summarizes some of the most recent membrane applications for the fractionation of food bioactive oligosaccharides. These approaches will be discussed in the following subsections according to the applied membrane separation technology.

13.2.1. Ultrafiltration (UF)

UF is a well-established membrane separation process for the purification of oligosaccharides from high molecular weight enzymes and polysaccharides. In general terms, there are a considerable number of studies addressing the fractionation of GOS, fructooligosaccharides (FOS) and xylooligosaccharides (XOS) by UF, which is in accordance with the amount of available data supporting their bioactivity. Thus, UF has

shown to be a very useful technique for the continuous enzymatic production of GOS using a cross-flow ultrafiltration membrane reactor. In general terms, this strategy leads to the continuous removal of the GOS (along with water, some substrate and simple sugar by-products) while the enzyme is retained by the membrane and returned to the reactor. As an example, **Figure 13.2** shows a laboratory scale membrane-assisted reactor system. The main advantages of this continuous process rely on issues related to product inhibition (by eliminating the monosaccharide fraction which inhibits the β-galactosidase activity), contamination in multi-step processes, and the reuse of enzyme without the need for its deactivation, among others (Czermak *et al.* 2004).

Foda & Lopez-Leiva (2000) optimized the production of GOS from whey permeate containing different concentrations of lactose and β-galactosidase from *Kluyveromyces lactis* by using either a laboratory scale (Amicon stirred cell, 41.8 cm^2 effective area) or a pilot plant-scale membrane reactors. The largest yield of GOS (concentration of GOS/initial lactose concentration) was 31% for whey UF permeate containing initially 20% lactose and 0.5% of the enzyme when the pilot plant scale membrane reactor, consisting of a UF-hollow fiber Romicon module (PM-10), with nominal molecular weight cut off (NMWCO) 10,000 and an effective area of 0.5 m^2, was used. A later study using the same enzyme and comparing the batch production in a stirred-tank reactor with the continuous production in a laboratory-scale reactor system fitted with 10-kDa NMWCO composite regenerated cellulose UF membrane was carried out (Chockchaisawasdee *et al.* 2005). The continuous process provided better productivity of GOS than the batch process, which was attributed to the ability to maintain a permanent state, without loss of enzyme activity. In a similar study, Petzelbauer *et al*. (2002) used either a batch reactor or a continuous stirred-tank reactor coupled to a 10-kDa cross-flow UF module having polyethersulfone membranes which were stable for

more than 10 weeks at a temperature of up to 80 ºC for the production of GOS with two thermostable β-galactosidases from *Sulfolobus solfataricus* and *Pyrococcus furiosus*, respectively. A cut-off of 10 kDa was required to retain β-galactosidases to an extent greater than 99%, allowing their reuse for the continuous mode of operation. The comparison of the production of GOS in batch wise and continuous mode of operation revealed that the amount of GOS produced by the β-galactosidase from *S. solfataricus* was significantly higher (between 3- and 1.3-fold) in the continuous than in the batch reactor probably due to a more efficient transfer of D-galactosyl residues to D-glucose and lactose, and a smaller extent of secondary degradation of GOS in the former. In addition, differences in terms of composition of the individual GOS were also observed.

Czermak *et al*. (2004) described the continuous production of GOS from lactose in a continuous membrane-assisted reactor fitted with either a polymeric (NMWCO 50,000 Da) or a ceramic membrane (NMWCO 20,000 Da) by using different β-galactosidases, number of feed concentrations and average residence times. The variation of these parameters had a notable influence on the yield and composition of the GOS fraction. In consequence, the maximum achieved GOS concentration was over 40% (w/w) with an average residence of 1 h and a feed lactose concentration of 31% (w/w). Pocedicova *et al*. (2010) compared the production of GOS using three different starting substrates (i.e., a buffered solution of lactose, recombined whey and UF-permeate) with similar concentration of lactose (200 g/L) in batch and continuous membrane reactor with UF ceramic membrane (150 kDa). The highest yields in GOS were obtained with recombined whey, followed by UF-permeate and lactose in buffer.

In addition to the use of native enzymes, UF also allows the option to use immobilized enzymes. Consequently, Matella *et al*. (2006) compared the production of

GOS in a recycled free-enzyme UF and in a recycled immobilized-enzyme systems. Both systems showed very similar maximum GOS formation (20-22%, w/w) after 15-17 min of reaction with a β-galactosidase from *Aspergillus oryzae*. Ebrahimi *et al*. (2010) applied a novel two-stage integrated ceramic membrane reactor system to physically immobilize β-galactosidase from *K. lactis* for the continuous production of GOS as it is illustrated in **Figure 13.3**. The UF membrane had a NMWCO of 20,000 Da and consisted of a support layer (Al_2O_3) with large pores and a low-pressure decay and one separation layer (TiO_2) to control the permeation flux. This system was very efficient in producing GOS since the maximum formation exceeded 38% (w/w) when an average residence time of 23.5 min, a transmembrane pressure of 2 bar and an initial lactose concentration of 30% (w/w) were applied.

UF in diafiltration mode with a regenerated cellulose membrane (NMWCO 1,000 Da) was used for the successful fractionation of a commercial FOS solution (Raftilose P95) consisting of a mixture of oligosaccharides with DPs from 2 to 7 (as previously mentioned in Chapter 4), as well as small amounts of fructose, glucose and sucrose (Montilla *et al.* 2006). Results indicated that diafiltration greatly reduced the concentration of mono-, di- and trisaccharides, whereas levels of FOS with DPs larger than 4 significantly increased in the mixture. Likewise, the reduction of mono- and disaccharide fraction in the diafiltered FOS was larger than the reduction of the trisaccharide concentration. In contrast, diafiltration mode using NMWCO 500 and 1,000 Da membranes on the same system than above was not suitable for GOS fractionation (Hernández *et al*. 2009). These data suggests that not only the oligosaccharide size but also the structure and, consequently, the conformation might play a role in membrane fractionation.

XOS derived from different sources (please, see Chapter 7 for more information about production and bioactivity) have also been purified by UF. Crude XOS require extensive purification processes before their use as functional food ingredients due to the presence of large amounts of undesirable products, such as lignin-related phenolics, non-saccharide fractions derived from extractions, digestible monosaccharides and their dehydration and condensation products, or ashes, among others. Moreover, XOS normally show a broad distribution of molar mass regardless of the reaction conditions (Nabarlatz *et al.* 2007). Therefore, membrane separation processes could be a suitable solution for the production of XOS with high purity and well-controlled molar mass distribution following their separation from higher molar mass products, as well as the fractionation of XOS with different DPs. In this context, Nabarlatz *et al.* (2007) used four commercial thin-film polymeric UF membranes with different NMWCO (i.e., 1.0, 2.5, 3.5 and 8.0 kDa) at pressures from 2.6 to 9 bar for the purification of XOS obtained from autohydrolysis of almond shells. Results indicated that the selectivity towards the permeation of lignin-related product impurities over oligosaccharide-related products was better at low fluxes of permeate and with membranes of low NMWCO. In another study, a two-step membrane processing based on UF by using 10 and 3 kDa membranes was employed for the fractionation of XOS derived from the enzymatic or acid hydrolysis of tobacco stalk (Akpinar *et al.* 2010). The first UF step which employed a 10 kDa NMWCO membrane was useful for removing the high molecular weight polysaccharides and proteins from the oligosaccharides which had a recovery of 90% (w/w). Then, the oligosaccharide syrup permeate was subjected to a second UF step having a 3 kDa cut off membrane and whose retentate was mainly composed of XOS with a DP above 6 whereas the permeate contained mainly xylobiose and xylotriose, followed by xylotetraose and minor amounts of xylopentaose, xylose and high DP

compounds. Overall, 71% (w/w) of XOS with DP between 2 and 6 were present in the reaction hydrolyzate at the end of the two-step UF process. Finally, Wang *et al*. (2011) used UF with a commercial membrane with a NMWCO of 5 kDa to efficiently remove xylan from XOS.

In another study, UF and ethanol precipitation were compared for the separation and fractionation of arabinoxylooligosaccharides (AXOS) from their monomeric building blocks, arabinose and xylose, and polysaccharides (Swennen *et al.* 2005). One step UF using three commercial membranes with NMWCO of 5, 10 and 30 kDa, as well as two step UF (10 and 30 kDa) processes were assayed. In general terms, although the fractions obtained by UF were more heterogeneous and polydisperse than the ones obtained with ethanol precipitation, AXOS fractions with similar DP and degree of substitution were obtained with both methods.

Finally, UF separation has also been successfully used for the purification of oligosaccharides from different origins, such as pectic oligosaccharides (Olano-Martin *et al.* 2001), hemicellulose-derived oligosaccharides (González-Muñoz *et al.* 2011), chicory and dahlia fructans (Moerman *et al.* 2004), isomaltooligosaccharides (IMOS, Zhang *et al.* 2010), or lactose-derived oligosaccharides from goat milk (Martinez-Ferez *et al.* 2009).

13.2.2. Nanofiltration (NF)

NF technology is a useful technique, especially, when UF does not offer sufficient rejection of low molecular weight solutes. Consequently, NF can be a better membrane technology to obtain larger concentrations of oligosaccharides than UF by increasing the rejection of mono- and, at a lesser extent, disaccharides. The main operating

differences between NF and UF are the typical membrane pore size (2 nm *vs*. 1-50 nm) and operating pressure (10-50 bar *vs*. 1-15 bar). In addition, the NMWCO of NF membranes normally lies in the range 200-1,000 Da (Tsuru *et al.* 2000), combining UF and RO separation properties (Goulas *et al.* 2002).

Goulas *et al*. (2002) studied five different flat sheet membranes with capacity of operating at maximum pressures between 20 and 35 bar in a cross-flow system for the fractionation of a model solution containing fructose, sucrose and raffinose, and of a commercial GOS mixture. Under optimal conditions, continuous diafiltration gave rise to satisfactory yield values for the GOS mixture (14-18% monosaccharides, 59-89% disaccharides and 81-98% oligosaccharides) indicating that the removal of monosaccharides was very efficient with minor losses of the oligosaccharide content of the mixture. Furthermore, these authors established that the rejection factors of the sugars were largely dependent on pressure, sugar concentration of the feed and filtration temperature. Similar purification levels of nanofiltered GOS were obtained in a subsequent study performed by the same authors, whilst higher losses of di- and oligosaccharides were reported by applying UF (Goulas *et al*. 2003).

Feng *et al*. (2009) recently used four commercial spiral wound NF membranes for the fractionation of GOS mixtures at low pressure (up to 8 bar), in order to avoid fouling due to the compaction of the membrane layer by operating at high pressure (Martinez-Ferez *et al.* 2006a). Moreover, the main advantage of this type of membranes is their feasibility to scale-up the filtration process, which could find an immediate application for the industry. Under optimum conditions, these authors reported 90.5% and 52.5% of rejection levels of monosaccharides and lactose, respectively, whereas 54.5% of the oligosaccharide purity was obtained (1.5 times of the raw material). Botelho-Cunha *et al*. (2010) observed a similar rejection pattern for NF fractionation of

a GOS mixture at two different temperatures (25 and 40 ºC). Thus, GOS-trisaccharides were totally retained and pressure-independent, while rejection coefficients of di- and monosaccharides increased with the effective transmembrane pressure applied. Interestingly, these authors observed different rejection levels for lactose and GOS-disaccharides, indicating that rejection of neutral solutes by NF is not only dependent on simple sieving effects, but differences in the conformation carbohydrate due to the nature of the involved glycosidic linkages may also have an effect in the selectivity of the NF process. Recently, Sen *et al*. (2012) have developed a rotating disk membrane bioreactor (RDMBR) to alleviate the problem of membrane fouling by giving rotational motion to the membrane. This novel bioreactor was successfully applied for the production of GOS by immobilized β-galactosidase on NF membrane giving rise to GOS yield and purity of 67.4% and 80.2%, respectively. These values were above to those obtained in batch mode followed by diafiltration-assisted NF.

NF in a continuous diafiltration mode has also been successfully applied for the purification of lactulose syrup by removing more than 96.5% of NaCl and the chemical catalyst (H_3BO_3) with only 11% loss of disaccharides (i.e., lactulose and lactose) (*Zhang et al.* 2011).

A forced-flow membrane reactor (FFMER) in which a β-fructofuronidase was immobilized to different porous ceramic NF membranes was applied for the continuous production of FOS (Nishizawa *et al.* 2000). The reason to use ceramic membranes was based on their resistance to heat and chemical agents, as well as their surface can be modified easily by silanization allowing the enzyme immobilization. The saccharide composition of FOS was a function of the permeate flux, which was easily controlled by pressure. Overall, the FOS percentage in the saccharide composition with the FFMER system ranged from 54.2% to 55.9%, although this percentage was increased up to

57.2% by performing a two-stage NF. Li *et al*. (2004) also successfully applied NF for the purification of FOS in constant volume diafiltration (CVD) and variable volume diafiltration (VVD) modes, where the dilution water flux is not equal to that of permeate, to yield in both cases FOS syrup purity above 90%. These results revealed that the relationships between purity and yield of FOS were independent on the dilution ratio. These same authors determined in a later study that the factors which affected FOS transport inside NF membrane were pressure gradient, steric hindrance and wall friction by using an extended pore model (Li *et al.* 2005). More recently, Kuhn *et al*. (2010) developed a process that included two NF stages with the same membrane to purify FOS also containing glucose, fructose and sucrose. The first stage was a diafiltration process which served to reduce the effects of osmotic pressure and membrane fouling, and the resulting permeate was nanofiltered to lead FOS with purities above 90% and yields around 80%. Similar FOS yields were obtained by the same authors using a tangential membrane cell filtration in a diafiltration mode (Kuhn *et al.* 2011).

Purification of XOS has also been addressed by NF with a series of studies published during the last decade. Yuan *et al*. (2004) reported a pilot-plant production of XOS from corncob meal based on a downstream process characterized by a steaming treatment to extract xylan (optimum conditions were 160 ºC and 1.5 h), followed by its enzymatic hydrolysis during 5 h, flocculation, ion exchange desalination and, finally, a NF step to purify and concentrate XOS offering the advantages of energy-saving and partial removal of monosaccharides. Afterwards, charcoal adsorption and vacuum evaporation were also applied. The total yield of XOS based on the dry corncob meal was 16.9% and the XOS syrup contained 74.5% of xylobiose (DP 2) and xylotriose (DP 3). Vegas *et al*. (2008) compared the fractionation and purification of XOS from monosaccharides

and other low molar mass materials, obtained from rice husk xylan with a polymeric tubular UF (NMWCO 4 kDa) membrane and with a ceramic monolithic NF (1 kDa) membrane. Results showed that the UF membrane gave the best fractionation results, but lower recovery yields than the NF membrane. These authors determined that an increase in transmembrane pressure resulted in improved XOS recovery, at the expense of a lower purity. Likewise, the final retentate from the NF process was further subjected to ion exchange and extraction with ethyl acetate which led to XOS with purity over 91% and with an overall yield of 71%. This downstream processing was previously optimized by the same research group (Vegas *et al.* 2006). Gullón *et al*. (2008) used NF for refining of autohydrolysis liquors, prior to the enzymatic hydrolysis with a commercial xylanase, and a subsequent ion exchange step with an anionic resin to purify XOS from rice husks. In a similar way, a processing strategy for XOS manufacture and purification from industrial solid wastes of malting industries (i.e., barley husks, spent grains and grain fragments) based on a double hydrothermal processing, double NF process (in diafiltration and concentration modes) with enzymatic hydrolysis and/or ion exchange and/or freeze drying has been developed (Gullón *et al.* 2011). Recently, Zhao *et al*. (2012) purified XOS from syrup by a discontinuous diafiltration process using a TFC spiral-wound NF membrane.

13.2.3. Combined membrane processing

The combination of several membrane separation technologies can be a powerful strategy to improve the yield and purity of oligosaccharide mixtures. Thus, application of NF is an attractive separation process for both desalting and concentration of UF permeate collected in dairy industry, by ensuring the recovery of lactose in abundance

for producing GOS (Rice *et al.* 2009). As examples, combination of two stage UF-NF process with tubular ceramic membranes of NMWCO 50 and 1 kDa, respectively, has been used for the recovery of oligosaccharides in caprine milk leading to a final retentate containing more than 80% of the original oligosaccharide content and virtually free of lactose (less than 4%), protein and salts (Martinez-Ferez *et al.* 2006a; Martinez-Ferez *et al.* 2006b). Briefly, the first step was useful to separate oligosaccharides from proteins, and the resulting permeate was subjected to NF to subsequently elute salts and lactose.

Kamada *et al*. (2002) also demonstrated the effectiveness of combined membrane processing with UF and NF for purifying and concentrating FOS from chicory rootstock. Thus, after the first UF step carried out with a polysulfone membrane of NMWCO of 20 kDa, 80.7% of the saccharides in the initial feed were recovered in the total permeate. The permeate was subjected to NF by using four different membranes made of polyamide composite with nominal salt rejection of 65, 30, 10 and 3%, respectively. Among all of them, the membrane with the nominal salt rejection of 30% exhibited the most suitable rejection for purifying FOS. Thus, under the optimized conditions, mono- and disaccharides (mainly glucose, fructose and sucrose) were preferentially removed from the retentate while FOS of DP $\geq$3 were retained and concentrated to give a final composition of the NF permeate as follows: 7.7% of monosaccharides, 43.3% of disaccharides and 42.7% of oligosaccharides with DP ranging from 3 to 10.

Gullón *et al*. (2010) used UF membranes to successfully refine XOS produced by hydrothermal processing of rice husks and to further purify them in combination with ion exchange. For the refining step, the autohydrolysis liquors from rice husks were ultrafiltered in diafiltration mode using a 1 kDa NMWCO membrane, then, nanofiltered

(concentration mode) also with a 1 kDa NMWCO and the resulting retentate was subjected to enzymatic treatment with commercial endoxylanases to reduce the average molar mass. The hydrolyzate was ultrafiltered in concentration mode with a 15 kDa NMWCO membrane and the permeate was further treated with an anionic exchange resin and, finally, freeze-dried. The percentages of recovery in the final product with respect to the starting material (i.e., autohydrolysis liquors) were: 4.9-14.1% for monosaccharides, 38% for glucooligosaccharides, 37.2% for XOS, 29% for arabinosyl moieties linked to oligosaccharides, 31.3% for acetyl groups, 26.7% for galacturonic acid equivalent and 2.3% for other non-saccharide and nonvolatile compounds. In this context, it can be seen that the combination of membrane processing with other purification steps (such as ion exchange or solvent extraction) is also normally used to improve the efficacy of the fractionation of complex oligosaccharide mixtures. In another study, Rivas *et al*. (2012) also employed a combination of UF and NF using regenerated cellulose membranes of 5 and 1 kDa NMWCO within a more complex strategy scheme also involving double hydrothermal processing to produce bifidogenic oligosaccharides derived from wood mannan.

Combination of preliminary UF and either RO or NF was also used for the recovery of oligosaccharides from steamed soybean waste water in tofu (soybean protein curd) processing giving rise to moderate yields of total oligosaccharides of 10% (w/v) and 22% (w/v), respectively (Matsubara *et al.* 1996).

To finish, Das *et al*. (2011) have reported the production of GOS from whey permeate with a purity of 77-78% using three-step membrane separation techniques: the first two were the UF step with polyethersulfone membranes of 50 kDa and 5 kDa NMWCO, followed by NF in a diafiltration mode (TFC-SR2 membrane, 400 Da NMWCO). The UF permeate was mainly comprised of unreacted lactose and produced

glucose, galactose and GOS. NF step was carried out at 1 MPa transmembrane pressure (TMP) at 25 ºC and was useful to fractionate GOS from mono- and disaccharides. Also, these authors indicated that the RO process used prior to hydrolysis was useful to remove some minerals from whey and might have increased the yield and efficiency of the whole process.

13.3. Chromatographic techniques

Size exclusion and ion exchange are by far the most used chromatographic techniques for the semi- or preparative fractionation of food bioactive oligosaccharides. Other techniques such as simulated moving bed or activated charcoal which are characterized by their feasibility of scale up for industrial applications will also be described in this section.

13.3.1. Size exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) is a powerful technique that allows separation of oligosaccharides in a size-dependent mode when a solution flows through a packed bed of porous packing that are available with a wide range of pore volumes. In consequence, separation of carbohydrates depends on the ratio of their molecular dimensions and the average diameter of the pores. In general, SEC has commonly been used for the separation of oligosaccharides homologous series. Semi-preparative or preparative High Performance Size Exclusion Chromatography (HPSEC) can be also used for the fractionation of carbohydrates depending on the amount of carbohydrate required.

Gels operating at low temperatures (around 25-30 ºC) such as dextran gels marketed under the trade name Sephadex (GE Healthcare, Uppsala, Sweden), agarose gels (trade name BioGel-A from Bio-Rad) and Sepharose (GE Healthcare) or polyacrilamide gels (BioGel-P from Bio-Rad), among others, have been used (Churms, 2002). Occasionally higher temperatures (35-55 ºC) can be applied on specific polysaccharide or dextran gels to decrease the interactions between the oligosaccharides and the gel matrix, improving the resolution, although the life of the packing becomes shorter. Water is the mobile phase chosen for the elution of carbohydrates.

Regarding detection, although several advances in analytical techniques have emerged in the lasts years, differential refractometers are still the most common detectors used in SEC, mainly considering the advantages of coupling, direct detection of carbohydrates and relatively low cost.

Several authors have used SEC to remove digestible carbohydrates (which account around 45-50% of the product) from commercial GOS mixtures using polyacrylamide gels (Searle *et al.* 2010) or dextran gels (Shoaf *et al*. 2006; Huebner *et al.* 2007) to further study their anti-adhesive and/or prebiotic activities. Different DP fractions were obtained and combined to create the GOS equivalent to that of commercial products. Compared with other fractionation techniques (diafiltration, activated charcoal treatment and yeast treatment), SEC has shown to be the most appropriated to obtain different DP GOS fractions (up to DP8) with the highest purity (almost 100% for each DP fraction) and recovery (between 81-92%) (Hernández *et al.* 2009). Fractionation into different DPs of food oligosaccharides of different nature, such as glucooligosaccharides (Sanz *et al*. 2005), gentiobiose- (Sanz *et al.* 2006), raffinose- (Hernández-Hernández, *et al*. 2011) and cellobiose-derived oligosaccharides (Ruiz-Matute *et al*. 2011), have been performed to evaluate the influence of their molecular

weight in the effect on human gut bacteria. In general, purest fractions were obtained for lower DP oligosaccharides (DP3-DP5), whilst fractions of higher molecular weight could not be completely purified as it can be observed in the TLC densitogram of alternansucrase maltose-acceptor oligosaccharide fraction obtained after separation by SEC using a polyacrilamide gel (**Figure 13.4**, Sanz *et al.* 2005).

SEC is also used to purify naturally occurring oligosaccharides from different matrices such as FOS from garlic extracts (Zhang *et al*. 2012), neutral and pectic oligosaccharides from olive by-products (Lama-Muñoz *et al*. 2012), etc.

Different size exclusion columns connected in series have been used to guarantee the fractionation and purification of carbohydrates such as galactoglucomannans extracted from *Picea abies* (Lundqvist *et al*. (2003) or xylooligosaccharides (Sun *et al*. 2002).

Annular SEC is a system consisting of two concentric cylinders forming an annulus into which the stationary phase is packed. Finke *et al*. (2002) compared this system, with the fixed bed conventional gel chromatography for the fractionation of fructans, and the productivity (grams of carbohydrate separated per hour) of annular chromatography was 25-fold higher than that of the conventional method, thus, facilitating the fractionation at preparative scale.

Overall, SEC is an effective technique for the fractionation of carbohydrates which are obtained at high purity and yields, however, its main drawback is that it is a time consuming technique and target carbohydrates are recovered in high volumes of solvent which should be removed after the process. To overcome these problems different approaches have been proposed such as the development of rapid methods based on the use of different materials, e.g. Superdex-type columns (GE Healthcare) based on highly cross-linked porous agarose beds to which dextran has been covalently bonded

(Knutsen *et al*, 2001) or the use of flash liquid chromatography (Flash LC) which use glass columns and gas pressure-driven flow between 0 and 2 bar (Strum *et al.* 2012).

13.3.2. Ion Exchange

Most food carbohydrates are weak electrolytes and, in consequence, they normally show little interaction in its natural form with ion exchange resins in an aqueous medium (Sanz & Martínez-Castro, 2007). However, acidic oligosaccharides, such as sialic or oligogalacturonic acids, are negatively charged at low pH and they can be efficiently fractionated by anion-exchange chromatography on a semi-preparative or preparative scale. Thus, Smith *et al*. (1978) reported the large-scale isolation of seven sialyl oligosaccharide fractions from human milk by anion-exchange chromatography on a DEAE-cellulose column (45 x 1.5 cm) in acetic–pyridine buffers, pH 5.4. More recently, Finke *et al*. (1999) also fractionated human milk oligosaccharides (HMOs) into neutral and acidic oligosaccharides by anion-exchange chromatography on an AG 1-X2 column (30 x 4.4 cm) with 250 mM ammonium acetate, pH 5.0 After desalting, the neutral and acidic fractions were further separated by SEC. Findings from this work strengthened the great structural complexity of HMOs (the reader can see Chapter 1 for more information on HMOs).

Oligogalacturonic acids have been largely isolated by semi-preparative or preparative anion-exchange chromatography. These oligosaccharides are derived from the depolymerization of polygalacturonic acids which are major components of cell wall polysaccharides (pectins) in plant tissues and can be present in dietary vegetables and fruits (the reader could find more information on this topic in Chapter 6). The preparative-scale isolation of oligogalacturonic acids has been traditionally performed

by ambient-pressure strong anion-exchange resins including Dowex I-X8 (Nagel & Wilson 1969), AG 1-X8 (Dave *et al*. 1976), DEAE-Sephadex A-50 (Liu & Luh, 1978), DEAE-Sephadex A-25 (Jin & West 1984; Suzuki *et al*. 2002), QAE-Sephadex (A-25-120) (Davis *et al*. 1986), AG MP-1 (Doner *et al*. 1988). These methods were time consuming, labor intensive, and generally lack adequate resolution for oligogalacturonic acids larger than a DP of 7, although in some cases oligogalacturonic acids of up to DP 17 could be detected. To overcome these drawbacks, Hotchkiss *et al*. (1991) developed a faster and more efficient preparative HPLC method than those previously reported by using a weak anion-exchange aminopropylsilica gel column (25 x 2.14 cm) which allowed the successful isolation of oligogalacturonic acids up to a DP of 7 in gram quantities. Later on, Spiro *et al*. (1993) purified oligogalacturonic acids with a DP between 10 and 15 (around 70 mg of each fraction) using Q-Sepharose (50 x 2.2 cm) fast-flow anion-exchange chromatography followed by semipreparative CarboPac PA1 (25 x 0.9 cm) high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The semipreparative CarboPac PA1 column was also used for the isolation of oligosalacturonic acids up to DP 5 (Zhan *et al*., 1998), as well as for rhamnogalacturonan oligosaccharides (Zhan *et al*., 1998; Schols *et al*., 1994; An *et al*., 1994). Also, Melotto *et al*. (1994) used the preparative CarboPac PA1 column (28 x 3.5 cm) to successfully isolate rhamnogalacturonan oligosaccharides. Later on, Hotchkiss *et al*. (2001) reported the isolation of multi-milligram quantities of oligogalacturonic acids up to DP 20 by high performance anion-exchange chromatography utilizing a preparative CarboPac PA1 column (25 x 2.1 cm) and a nonlinear potassium acetate (pH 7.5) gradient. Guillaumie *et al*. (2006) have also described the preparative separation of multi-milligram quantities of pectin oligogalacturonides with DPs from 5 to 19 and purity above 95% by using a strong

anion-exchange resin Source 15Q combined with ammonium formate buffer. A similar resin was used by Kabel *et al*. (2002) to separate neutral (acetylated) xylooligosaccharides from acidic (acetylated) xylooligosaccharides derived from hydrothermally treated Eucalyptus wood and brewery's spent grain in combination with SEC.

Moving on cation-exchange chromatography applications, these are normally employed for desalting solutions of oligosaccharides and data about fractionation of food bioactive oligosaccharides is scarce. Vente *et al*. (2005) investigated the influence of Ca^{2+}, Na^{+} and K^{+} as the cations of the strong acid cation exchange resin Dowex 50WX4-400 on the chromatographic removal of monosaccharides from disaccharides. Results indicated that K^{+} loaded resin had a stronger adsorption of sugars than the Na^{+} loaded resin. The lower retention of disaccharides was explained by the size exclusion mechanism, whereas difference in retention within the monosaccharide fraction was related to the number of equatorial-axial oriented sugar OH groups for complexation with the cation. These authors concluded that the K^{+} ion was the most suitable cation for separation of glucose from oligosaccharides whilst Ca^{2+} cation was the best choice for removal of fructose from oligosaccharides. Early on, Keisuke & Tamura (1988) registered a patent based on a method for producing GOS wherein they used a preparative scale chromatography column with a strong acid cation exchange resin, i.e. Unibead UBK-530, in Na-form, heated at 60 °C for the purification of GOS. The separation of the components of the GOS mixture was driven by the molecular size exclusion effect, and fractions containing high concentrations of oligosaccharides, high concentrations of lactose, and high concentrations of monosaccharides were respectively collected from the eluates from the column. Sinclair *et al*. (2009) adapted

this preparative method also for the purification of GOS although these authors exchanged the Na^+ ion with a K^+ ion.

13.3.3. Simulated moving bed chromatography

Simulated moving bed (SMB) chromatography was developed in the early 1960s (Broughton & Gerhold 1961) and has since then been widely used by the sugar industry for the production of several mono- and oligosaccharides at the multi-ton scale (Heuer *et al*., 1998; Nicoud 1998; Schulte & Strube 2001). Briefly, SMB is a chromatographic technique based on a flow of liquid (mobile phase) moving countercurrent to a constant flow of solid (stationary phase). Countercurrent flow enhances the potential for the separation and, hence, makes the process more efficient, higher throughput and consumes less solvent (mobile phase) in comparison to traditional batch elution chromatography (Mihlbachler & Dapremont 2005). Given that providing a constant flow of solid is impractical in a production process, the solid instead is packed into several high pressure columns (in most cases 6-12) which are connected in series and between each of them four valves are placed which can be individually opened and closed. A recycling pump inside the column circle delivers the mobile phase through all columns, whilst two additional pumps constantly introduce the feed and fresh eluent (inlet streams) and two pumps withdraw (outlet streams) the raffinate enriched with the less retained component and the extract enriched with the more retained component (Heuer *et al*. 1998; Schulte & Strube 2001). The inlet and outlet position is switched at regular time intervals in the direction of the liquid flow, mimicking an apparent solid flow opposite to the direction of the liquid flow, simulating, thus, a countercurrent movement of columns. An adequate operation on SMB chromatography requires

computer simulations rather than empirical approaches, considering the great number of parameters, such as column diameter, column length, total column number, number of columns per section, inlet, outlet and recycle fluid flows and switch time interval, which has to be optimized (Schulte & Strube 2001).

The SMB chromatography is very versatile since it is applicable for all kinds of chromatography (Geisser *et al*. 2005), although the adsorbents are usually a combination of size-exclusion and ion exchange gels (Sanz & Martínez-Castro 2007). Thus, Kishihara *et al*. (1989) developed a large scale separation of palatinose and trehalulose using a simulated moving-bed adsorber by using as stationary phase a UBK 530 resin of Ca-form. SMB chromatography was also used to efficiently separate lactose from HMOs using two different stationary phases: ion-exchange chromatography (MCI gel, Ca^{2+} form), as well as size-exclusion chromatography (HW40C gel) (Geisser *et al*. 2005). The IEC gel (MCI) was used for ligand-exchange chromatography separating the molecules according to their size. Both stationary phases were suitable to separation of lactose, giving rise to similar results of the relative lactose content in the extract and raffinate. Thus, with HW40C gel, the raffinate had levels of <4% of lactose in comparison to the complex oligosaccharides (neutral and acidic oligosaccharides), whilst the extract showed >96% of the separated lactose. Using MCI gel, the content of lactose varied in the raffinate from 0 to 20% compared to HMOs, while in the extract it had a value of >96%. However, important differences were found in flow rates, lactose yield and, specially, in stability of gels during their regeneration. The SEC gel provided lower flow rates and yield of lactose, and it was much more stable for the continuous SMB process since the IEC gel teared and shrinked during its regeneration, making it incompatible with the need of constant conditions. In contrast, the SEC gel did not undergo any change during regeneration and was much more

suitable and comfortable for the separation of lactose from HMOs as compared to the IEC gel. More recently, SMB chromatography was applied to three different sugar separations: raffinose-sucrose, fructose-glucose and xylose-glucose for comparison purposes with fractionation methods based on membrane cascades with different NF and UF membranes at pilot scale (Vanneste *et al*. 2011). These authors concluded that both fractionation methods could be suitable to successfully separate all studied applications. Likewise, two IEC resins in the K^+ form (Dowex 50W-X2 and Dowex Monosphere 99K/320) were studied for purification of FOS (Nobre *et al*. 2010). Both IEC gels were useful to separate fructose, glucose, sucrose and FOS with different DP, although the Dowex Monosphere 99K/320 gel was found to be more suitable to work in the SMB system due to its greater resistance at high pressure.

13.3.4. Activated charcoal

The use of columns packed with charcoal (activated carbon) is a conventional method used for preparative separations of oligosaccharides, especially, since the work of Whistler and Durso (1950) who fractionated different mixtures of mono-, di- and trisaccharide (i.e., raffinose) by using a mixture of equal amounts of Darco G-60 and Celite 535. The development of several carbons for HPLC has been made (Knox & Gilbert 1978), and, currently, the graphitized carbon columns (GCC), as commented in Chapter 19, are used for preparative separation of neutral linear oligosaccharides, *N*-linked oligosaccharides, chito-oligosaccharides, sulfated oligosaccharides, oligosaccharide alditols, cyclodextrins and glycopeptides (Koizumi *et al*. 2002).

Retention of oligosaccharides on activated carbon is mainly driven by an adsorption mechanism and planar molecules are generally more retained than non-planar ones

(Koizumi *et al*., 2002). These columns are especially suitable for the fractionation of mixtures that contains carbohydrates of different DP. Thus, by applying an activated charcoal treatment (Darco G60) to a typical mixture of GOS, monosaccharides and lactose were removed by eluting them with ethanol at low concentrations in water solutions (between 1% and 15%, v/v), whilst GOS were desorpted by eluting it with ethanol at a high concentration (50%, v/v) (Hernández *et al*. 2009). This treatment revealed to be a rapid method to obtain considerable amounts, at gram scale, of GOS. However, the main disadvantages of activated charcoal treatments are that saccharides may not be sufficiently separated with respect to their sizes, leading to an incomplete removal of disaccharides and, if an attempt is made to obtain GOS with high purity, the yield could be poor. This was the case reported by Hernández *et al*. (2009) who obtained GOS of high purity with no presence of monosaccharides and lactose with 10% of ethanol, although the recovery of trisaccharides was very low. Akpinar & Penner (2008) successfully fractionated near gram quantities of pure cellooligosaccharides in the DP range of 3 to 7 with a charcoal celite column packed with equal amounts of Darco G-60 and celite 545 and using a water-ethanol gradient (0-45%). More recently, GCC Flash LC combined with real-time mass spectrometry detection has shown a great potential to enrich and separate milligram quantities of specific human and bovine milk oligosaccharides, as well as to eliminate abundant saccharide components in milk, such as lactose and lacto-*N*-tetraose, and fractionate isomeric forms for more detailed biological assays (Strum *et al*. 2012). These results could not be achieved by size exclusion columns or amine columns.

13.3.5. Other chromatographic techniques

Although much less frequently used than those described above, this subsection briefly describes other chromatographic techniques that have also been used for the semi- or preparative isolation of food bioactive oligosaccharides during the last decade.

Coulier *et al*. (2009) fractionated the disaccharide and trisaccharide fractions from a commercial prebiotic GOS mixture by preparative Hydrophilic Interaction Liquid Chromatography (HILIC), facilitating the subsequent characterization of these fractions by methylation analysis and NMR.

Akpinar *et al*. (2004) demonstrated the potential of using cellulose stationary phases for the fractionation of water-soluble cellooligosaccharides. These authors indicated that the order of elution of this type of oligosaccharides (with DP from 3 to 6) was correlated with their relative solubility in aqueous ethanol solvents, as the higher DP cellooligosaccharides were less soluble and had longer retention times. Later on, Akpinar & Penner (2008) found that cellulose affinity/partition chromatography was the least time consuming and most economical method for the preparation of cellooligosaccharides of DP 4 and 5 as compared to charcoal-celite adsorption, SEC or cation-exchange chromatography.

Many studies have also been made to fractionate oligosaccharides into structurally distinct groups by affinity chromatography by the use of immobilized lectin columns. However, these methods have been mainly applied to glycoconjugates using lectins form different nature (lentils, peas, mushrooms, etc). More information can be found in different reviews (Osawa & Tsuj 1987; Endo 1996).

Finally, the successful purification of FOS from a mixture also containing glucose, fructose and sucrose on zeolite fixed bed columns (using either one or two columns in series) has been reported (Kuhn & Filho 2010).

13.4. Fractionation techniques using solvents.

Although most of the methods and techniques indicated below are dedicated to the extraction of carbohydrates from different matrices, some applications regarding carbohydrate fractionation could be emphasized.

13.4.1. Selective solvent solubilities

It is well-known that differences in solubilities of carbohydrates in organic solvent result in the selective precipitation of some of them which can be easily separated from the extraction mixture. Nevertheless, literature in this field is scarce.

As mentioned in Chapter 9, ketoses such as tagatose and lactulose can be considered prebiotic and both are obtained by alkaline isomerization or by biological treatments of the corresponding aldose (galactose or lactose, respectively). Separation of ketoses from non-bioactive unreacted aldoses is required. Some studies have evaluated the solubilities of these carbohydrates in different alcohols and water-alcohol mixtures (Olano, 1979; Montañés *et al.* 2007). In general, higher solubilities of lactulose and tagatose than lactose and galactose in these solvents at different temperatures (22, 30 and 40 ºC) have been found; thermodynamic models were proposed to choose the best solvent to selectively purify these ketoses in mixture with other sugars (Montañés *et al.* 2007). However, these methods usually require high volumes of organic solvents and new alternatives are been developing.

13.4.2. Ionic liquids

Ionic liquids (IL) are organic salts with melting points lower than 100 ºC which consists of organic cations such as imidazolium, pyrrolidinium, pyridinium, etc, and

different inorganic or organic anions such as chloride, dycianamide, etc (Han & Row, 2012). They have negligible vapor pressure, chemical and thermal stability, non-flammability, high ionic conductivity and wide electrochemical potential (Welton 1999). Bearing in mind these properties, ILs are considered efficient and environmentally friendly solvents in chemistry and are chosen as new alternatives instead of classical organic solvents.

ILs are being extensively use in carbohydrate chemistry mainly for synthesis and catalysis (El Seoud *et al.* 2007). However, applications in food science are still scarce and mainly associated to the extraction of amino acids, proteins, azo dyes or contaminants among others (Ruiz-Aceituno *et al.* 2013).

A method based on the different sugar solubilities of fructose and glucose in ILs at room temperature has been patented and applied to the selective separation of these sugars in dates (Al Nashef *et al.* 2008). Although this procedure is not applied to the production of bioactive carbohydrates it can be used as a model for further investigation. 1,3-Dimethylimidazolium dimethylphosphate and 1-ethyl-3-methylimidazolium ethylsulfate were selected for separation of fructose and glucose, respectively. These ILs dissolve fructose and glucose in large quantities but at different proportions.

Only, preliminary studies for the purification of ketoses from aldoses using ILs as green solvents can be found in the literature (Carrero *et al.* 2013). Solubilities of lactulose, lactose, tagatose and galactose in different ILS such as 1-ethyl-3-methylimidazolium dicyanamide, 1-hexyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium methyl sulphate have been determined. In general, ketoses were more soluble than aldoses in ILs. These data could be used to selectively separate these

carbohydrates, as well as to further apply this method for the fractionation of different structural oligosaccharides.

Although ILs could be promising tools for the fractionation of bioactive carbohydrates; more studies to carefully evaluate their toxicity should be conducted before their industrial applications. Up to now, they should be treated as any other chemical with a limited data about toxicity and biodegradability (Zakrezewska *et al.* 2010)

13.4.3. Supercritical fluids extraction (SFE).

Fractionation of carbohydrates by SFE is also based on the different solubilities of carbohydrates in supercritical carbon dioxide (SC-CO_2) which are enhanced by the use of polar co-solvents. Therefore, in the recent years SC-CO_2 with different co-solvents (isopropanol, methanol, alcohol/water mixtures, etc) have been applied to selectively separate bioactive ketoses such as tagatose from mixtures with galactose (Montañés *et al.*2006) or lactulose from mixtures with different aldoses (Montañés *et al.* 2008). The efficiency in the recovery and purity of ketoses is highly dependent on the nature of the co-solvent which enhanced their solubility helping to their separation. The modifier flow rate is the main factor influencing sugars recovery. Moreover, although a high increase of the amount of total carbohydrate extracted is achieved when more polar co-solvents are used, selective extraction of the specific carbohydrates decreased, which accounted for the maximum amount of modifier added. Once the method is optimized, extraction purities higher than 90% of ketoses and recoveries higher than 75% are achieved (Montañés *et al.*2006). However, when this method is applied to the separation of lactulose from a commercial mixture (Duphalac®; Solvay, Fr) constituted also by

galactose, tagatose and lactose, the complete purification of lactulose in the extract was not achieved, considering the high amounts of galactose extracted (Montañés *et al.* 2008).

SC-CO_2 with different ethanol/water mixtures as co-solvents has been also applied to the fractionation of complex carbohydrate mixture according to their DP. A two-step SFE procedure using ethanol/water as co-solvent and the most suitable extraction conditions (including temperature, pressure and co-solvent flow rate) allowed the almost complete removal of monosaccharides and disaccharides from a mixture of prebiotic carbohydrates (GOS) (75% of purity and 94% recovery) (Montañés *et al.* 2009). Another method including a third SFE step was also developed to obtain trisaccharides with high purity (Montañés *et al.* 2010). These processes optimized at laboratory scale to fractionate mixtures of prebiotic carbohydrates have been scaled-up to an industrial level simulating its economic feasibility. These studies proved that the process could be considered profitable with a pay-out period of about 10 years (Montañés *et al.* 2012).

13.4.4. Pressurized Liquid Extraction (PLE).

As indicated in SFE, pressure can influence on the solubility of carbohydrates in different organic solvents. PLE systems work at high pressure and controlled temperatures and allow the use of solvents in subcritical conditions. Although, these systems have been widely used for non-polar analytes, limited applications have been developed until now for the fractionation of bioactive carbohydrates.

Different applications regarding extraction and enrichment of carbohydrates using PLE and mainly subcritical water extraction (SWE) can be found in the literature

(Rodriguez-Sánchez *et al*. 2010; Guan *et al.* 2010; Pronyk & Mazza 2012, Song *et al*. 2012). However, fractionation processes are limited. In this sense, pressurized low polarity water (PLPW) extractor was successfully used for the extraction and separation of hemicellulose, cellulose, lignin, and other phenolic compounds in flax shives. Hemicellulose and lignin were mainly separated at 170 ºC, pH 3.0 and a flow rate of 2.5 mL/min. A two-stage PLPW extraction (the first stage to remove hemicellulose and the second stage for delignification) was used to improve the purity and yield of cellulose (Kim & Mazza 2009).

The fractionation of different cereals (triticale, durum wheat, CPS wheat, feed barley, oats) and oilseeds (canola, mustard) straws using hydrothermal processing with PLPW (at 165 ºC with a flow rate of 115 mL/min and a solvent-to-solid ratio of 60 mL/g) has been also proposed (Pronyk and Mazza 2011). Separation of oligosaccharides and low molecular weight carbohydrates (liquid fractions) from polysaccharides (mainly glucans, galactans, mannans, etc) was successfully achieved.

Separation of lactulose from a mixture with lactose [70:30 (w/w)] has been proposed using PLE at 1500 psi for 30 min. Different temperatures (from 40 to 130 °C) and ethanol:water ratios (70:30, 80:20, 90:10, 95:5, and 100:0) as extraction solvent were assayed. The optimum extraction conditions were 40 °C and 70:30 ethanol:water. PLE extraction under the optimized conditions was also applied to purify lactulose from lactose in a mixture of synthesis. Advantages of this technique over classical methods were the short extraction time and the low solvent consumption (Ruiz-Matute *et al.* 2007).

A combination process of PLE with an in-cell packed adsorbent bed of activated charcoal was also proposed for the separation of monosaccharides in honey samples

before their oligosaccharide analysis (Ruiz-Matute *et al.* 2008). Two consecutive PLE cycles using 1:99 (v/v) ethanol/water for 5 min and 50:50 (v/v) ethanol/water for 10 min, respectively, both at 10 MPa and 40 °C were necessary to obtain enriched fractions of di- and trisaccharides (73% and 8% of total carbohydrate, respectively). This method was compared with other procedures (i.e., yeast treatment and activated charcoal extraction; **Figure 13.5**). As can be observed in the figure, all methods were useful for selective removal of monosaccharides from honey, yeast treatment being the most effective (from 593 mg/g to 5 mg/g). However, the recovery of di- and trisaccharides was higher when the PLE procedure was used (74% and 79%, respectively). Moreover, PLE reduced extraction time and solvent volumes.

13.4.5. Microwave Assisted Extraction (MWAE)

MWAE is based on heating solvents in contact with samples using microwave energy with the aim of improving the partitioning of the target compounds from the matrix into the solvent. MWAE is carried out in closed-vessels and also combines the effect of pressure and temperature. Up to know applications regarding separation of bioactive carbohydrates are scarce and are mainly focused on the fractionation of high molecular weight carbohydrates such as galactoglucomannans (Lundqvist *et al.* 2003).

MWAE treatments have been recently used to fractionate carbohydrates from the coffee residue matrix remaining after preparing the beverage (Passos *et al*, 2012). Fractionation of water-soluble polysaccharides (galactomannans, type II arabinogalactans, and cellulose), oligosaccharides (mannooligosaccharides) and monosaccharides (mannose) was achieved at 900 W, 200 ºC for 5 min and 15 bar. Yields of 29.0% of water soluble material were obtained when these conditions were

applied to a ratio of 1 g of dried coffee residue to 30 mL of water extraction. The relative amount of polysaccharides, oligosaccharides, and monosaccharides extracted accounted for 83.4, 12.8, and 3.4%, respectively. The increase of the ratio of coffee residue mass to volume of water (r:w) resulted in a decrease of the amount of polysaccharides while increasing the oligosaccharides and monosaccharides content. Different percentages of galactomannans and arabinogalactans were obtained depending on the r:w ratio.

Water-soluble hemicelluloses were extracted from spruce chips and fractionated on the basis of their DP and composition by microwave treatment (Lundqvist *et al.* 2002 and 2003). The spruce chips were milled and impregnated (soaked) with water and NaOH. After impregnation, wood material was heat treated at a predetermined temperature (180, 190, or 200 ºC) and residence time (2 and 5 min) in a microwave oven. Fractionation depended on the temperature used and mainly on the degree of impregnation of spruce chips in NaOH solutions of different concentrations. As an example, the highest mannan yield was obtained from water impregnated spruce chips at 190 ºC for 5 min (molecular weight of 3,800), whereas the highest DP carbohydrates (molecular weight of 14,000) were obtained at these conditions from impregnation with 2% NaOH (Lundqvist *et al.* 2003).

Although other works are focused on the extraction of carbohydrates assisted by microwave, optimization of extraction conditions results in a fractionation depending on the solubility of carbohydrates in the chosen solvent at the assayed temperatures. As an example, MWAE was applied for production of arabinoxylans from corn pericarp which is an industrial waste of corn starch production. Xylans were separated from cellulose heating at 176.5 °C, for 16 min and solid to liquid ratio 1/20 (g/mL), respectively. The

maximal yield attained 70.8% of carbohydrates with predominant production of xylo-oligosaccharides (Yoshida *et al.* 2010).

13.5. Microbiological and enzymatic techniques.

Microbiological techniques can be considered a selective method for the fractionation of carbohydrates based on enzymatic activities.

Saccharomyces cerevisiae has been used to remove monosaccharides (glucose and galactose) from GOS mixtures (Goulas *et al.* 2007) producing ethanol and CO_2. Fermentations took place at 30 ºC under stirring using 1 g of freeze-dried yeast (2.9 x10 ^{10} cfu/g) per 100 mL of solution of carbohydrate mixtures (450 mg/mL). The mixtures were successfully purified from glucose (92% of glucose removed) by fermentation with this yeast with no losses in the oligosaccharide content, however, only a small decrease on the galactose was observed (**Figure 13.6**). However, previous works described the removal of individual galactose by yeast (Yoon *et al*., 2003). This behavior could be due to the high amounts of glucose present in the mixture which led the yeast cells to metabolise it initially leaving galactose initially unaffected (Goulas *et al.* 2007). Once glucose was removed, galactose assimilation should start, but these authors justify the presence of this monosaccharide by the high levels of the produced ethanol (7.4% v/v) which inhibited the action of yeast. Later, Hernández *et al.* (2009) also applied this treatment (*S. cerevisiae*) to purify GOS oligosaccharides getting the complete removal of both glucose and galactose in only 10 h of incubation. They justified these positive results considering that the concentration of GOS used in their work was lower (200 mg/mL) than that used by Goulas *et al.* (2007) and consequently the level of ethanol produced could be lower and would not be toxic to yeast.

Li *et al*. (2008) also used *S. cerevisiae* L1 and *K. lactis* L3 to selectively remove monosaccharides form a GOS mixture produced by β-galactosidases, resulting in an increase of GOS purity from 28.7% to 39.4% and 97.5%, respectively.

Apart from the high efficiency of yeast treatment in the removal of monosaccharides, this procedure can also be performed directly on the synthesis mixtures without the need of significant dilutions (as in other techniques such as nanofiltration) (Goulas *et al*. 2007), it is low cost process, easily scalable for industrial uses. However, the main disadvantage of yeast treatment for the fractionation of GOS is the incapacity of the yeast cells to remove the disaccharides such as lactose (digestible carbohydrate) and also the production of some metabolic products during the fermentation, such as ethanol and trehalose (Hernández *et al.* 2009).

The use of *S. cerevisiae* for the enrichment of legume extracts in bioactive inositols (free inositols, methyl-inositols and glycosyl-inositols) have been also successfully use. The selective removal of interfering carbohydrates was achieved; incubation time (3-40 h) was highly dependent on the composition of the legume considered (Ruiz-Aceituno *et al.* 2012).

Other treatments based on the use of different enzymes have been also proposed for the fractionation of carbohydrates and the purification of bioactive oligosaccharides. In this sense, Splechtna *et al*. (2001) assayed a selective enzyme oxidation for GOS purification using fungal cellobiose dehydrogenase which displays an approximately 100-fold preference for reaction with lactose compared to reaction with GOS. Oxidation of lactose was coupled to reduction of 2,6-dichloro-indophenol which was added in catalytic concentrations. The oxidised redox mediator was regenerated continuously by fungal laccase-catalysed reduction of molecular oxygen into water. Ion exchange

chromatography was employed to remove lactobionic acid, other ions and monosaccharides (Maischberger *et al*. 2008)

On the other hand, immobilized cells of the bacterium *Z. mobilis* have been used to remove glucose, fructose and sucrose from different oligosaccharide mixtures (fructo-, malto-, isomalto-, gentio-, and inulin-oligosaccharides). These mono- and disaccharide were completely fermented within 12 h of incubation with no pH control or nutrient addition (Crittenden & Playne 2002).

13.6. Conclusions

Fractionation of carbohydrates is a required task considering the production of bioactive oligosaccharides and the analysis of complex carbohydrate mixtures. Although several advanced techniques have emerged for this purpose, classical methods are still effective and widely used. The main disadvantages are still the time consuming techniques and the lower yields of purified carbohydrates.

Most of the techniques success in the fractionation of oligosaccharide mixtures regarding their molecular weight (see **Table 13.2**), However, the fractionation of carbohydrates with the same DP but differing in their glycosidic linkages or monosaccharide composition is still a challenging task. Some attempts have been described for specific carbohydrates (i.e. PLE for fractionation of lactulose and lactose (Ruiz-Matute *et al*. 2007), but more developments are required to extend them for general applications. Noteworthy progresses in chromatographic and related techniques have been performed mainly at analytical scale, including the development of new column packing and/or support materials, advances in orthogonality of two-dimensional chromatographic separations and/or hyphenated techniques, which have allowed

improving the separation between structurally related carbohydrates. However, advances of carbohydrate fractionation at semi- or preparative scale have been primarily focused on reducing the treatment time and there is still necessity for improving the selectivity between oligosaccharides having the same DP. This is especially important in the case of bioactive oligosaccharides as it is mandatory to establish a clear relationship between the claimed beneficial function and structure in order to comply with regulatory requirements.

Acknowledgements

This work has been financed by Projects AGL2009-11909, AGL2011-27884 and Consolider Ingenio 2010 FUN-C-FOOD CSD2007-00063 from Ministerio de Ciencia e Innovación, Project S2009/AGR-1464, ANALISYC-II from Comunidad Autónoma of Madrid (Spain) and European funding from FEDER program and project AGR-7626 from Junta de Andalucía.

References

Akin, O., Temelli, F. & Koeseoglu, S. (2012) Membrane applications in functional foods and nutraceuticals. *Critical Reviews in Food Science and Nutrition* **52**, 347-371.

Akpinar, O., McGorrin, R.J. & Penner, M.H. (2004) Cellulose-based chromatography for cellooligosaccharide production. *Journal of Agricultural and Food Chemistry* **52**, 4144-4148.

Akpinar, O. & Penner, M.H. (2008) Preparation of cellooligosaccharides: Comparative study. *Journal of Food Agriculture & Environment* **6**, 55-61.

Akpinar, O., Erdogan, K., Bakir, U. & Yilmaz, L. (2010) Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. *LWT-Food Science and Technology* **43**, 119-125.

Al Nashef, I., Gaily, M.H., Al-Zahrani, S.M. & Abasaeed, A.E. (2011) Method for separating fructose and glucose. *United States Patent* **No. US 7,942,972 B2**.

An, J., Zhang, L., O'Neill, M.A., Albersheim, P. & Darvill, A.G. (1994) Isolation and structural characterization of endo-rhamnogalacturonase-generated fragments of the backbone of rhamnogalacturonan I. *Carbohydrate Research* **264**, 83–96.

Botelho-Cunha, V.A., Mateus, M., Petrus, J.C.C. & de Pinho, M.N. (2010) Tailoring the enzymatic synthesis and nanofiltration fractionation of galacto-oligosaccharides. *Biochemical Engineering Journal* **50**, 29-36.

Broughton, D.B. & Gerhold, C.G. (1961) Continuous sorption process employing fixed bed of sorbent and moving inlets and outlets. *US Patent* **No. 2 985 589**.

Carrero, C., Ruiz-Aceituno L., Moreno F.J., Sanz M.L. (2013) Preliminary study of the use of room temperature ionic liquids for the purification of lactulose. *Journal of Agriculture and Food Chemistry* (sent)

Chockchaisawasdee, S., Athanasopoulos, V.I., Niranjan, K. & Rastall, R.A. Synthesis of galacto-oligosaccha ride from lactose using beta-galactosidase from *Kluyveromyces lactis*: Studies on batch and continuous UF membrane-fitted bioreactors. *Biotechnology and Bioengineering* **89**, 434-443.

Churms, S.C. (2002) Modern size-exclusion chromatography of carbohydrates and glycoconjugates In *Carbohydrate analysis by modern chromatography and electrophoresis* (Ed. Z. El Rassi). Elsevier, Amsterdam pp 267-299.

Coulier, L., Timmermans, J., Bas, R., Van Den Dool, R., Haaksman, I., Klarenbeck, B., Slaghek, T. & Van Dongen, W. (2009) In-depth characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques. *Journal of Agricultural and Food Chemistry* **57**, 8488-8495.

Crittenden, R.G. & Playne, M.J. (2002) Purification of food-grade oligosaccharides using immobilised cells of *Zymomonas mobilis*. *Applied Microbiology and Biotechnology* **58**, 297–302.

Czermak, P., Ebrahimi, M., Grau, K., Ketz, S., Sawatzki, G. & Pfromm, P.H. (2004) Membrane-assisted enzymatic production of galactosyl-oligosaccharides from lactose in a continuous process. J*ournal of Membrane Science* **232**, 85-91.

Das, R., Sen, D., Sarkar, A., Bhattacharyya, S. & Bhattacharjee, C. (2011) A Comparative study on the production of galacto-oligosaccharide from whey permeate in

recycle membrane reactor and in enzymatic batch reactor. *Industrial & Engineering Chemistry Research* **50**, 806-816.

Dave, B.A., Vaughn, A.G. & Patel, I.B. (1976) Preparation, separation and degradation of oligouronides produced by the polygalacturonic acid transeliminase of Bacillus pumilus. *Journal of Chromatography* **116**, 395–405.

Davis K.R., Darvill, A.G., Albersheim, P. & Dell, A. (1986) Host-Pathogen Interactions : XXIX. Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase are elicitors of phytoalexins in soybean. *Plant Physiology* **80**, 568-577.

Doner, L.W., Irwin, P.L. & Kurantz, M.J. (1988) Preparative chromatography of oligogalacturonic acids. *Journal of Chromatography* **449**, 229-239.

Ebrahimi, M., Placido, L., Engel, L., Ashaghi. K.S. & Czermak, P. (2010) A novel ceramic membrane reactor system for the continuous enzymatic synthesis of oligosaccharides. *Desalination* **250**, 1105-1108.

El Seoud, O.A., Koschella, A., Fidale, L.C., Dorn, S. & Heinze T. (2007) Applications of ionic liquids in carbohydrate chemistry: a window of opportunities. *Biomacromolecules* **8**: 2629-2647.

Endo, T. (1996) Fractionation of glycoprotein-derived oligosaccharides by affinity chromatography using immobilized lectin columns. *Journal of Chromatography A* 720, 251-261.

Feng, Y.M., Chang, X.L., Wang, W.H. & Ma, R.Y. (2009) Separation of galacto-oligosaccharides mixture by nanofiltration. *Journal of the Taiwan Institute of Chemical Engineers* **40**, 326-332.

Finke, B., Stahl, B., Pfenninger, A., Karas, M., Daniel, H. & Sawatzki, G. (1999) Analysis of high-molecular-weight oligosaccharides from human milk by liquid chromatography and MALDI-MS. *Analytical Chemistry* **71**, 3755-3762.

Finke, B., Stahl, B., Pritschet, M., Facius, D., Wolfgang, J. & Boehm, G. (2002) Preparative continuous annular chromatography (P-CAC) enables the large-scale fractionation of fructans. *Journal of Agricultural and Food Chemistry* **50**, 4743-4748.

Foda, M.I. & Lopez-Leiva, M. (2000) Continuous production of oligosaccharides from whey using a membrane reactor. *Process Biochemistry* **35**, 581-587.

Geisser, A., Hendrich, T., Boehm, G. & Stahl, B. (2005) Separation of lactose from human milk oligosaccharides with simulated moving bed chromatography. *Journal of Chromatography A* **1092**, 17-23.

González-Muñoz, M.J., Santos, V. & Parajo, J.C. (2011) Purification of oligosaccharides obtained from Pinus pinaster hemicelluloses by diafiltration. *Desalination and Water Treatment* **27**, 48-53.

Goulas, A. K., Kapasakalidis, P. G., Sinclair, H. R., Rastall, R. & Grandison, A. S. (2002) Purification of oligosaccharides nanofiltration. *Journal of Membrane Science* **209**, 321–335.

Goulas, A.K., Grandison, A.S. & Rastall, R.A. (2003) Fractionation of oligosaccharides by nanofiltration. *Journal of the Science of Food and Agriculture* **83**, 675-680.

Goulas, A., Tzortzis, G. & Gibson, G.R. (2007) Development of a process for the production and purification of α- and β-galactooligosaccharides from *Bifidobacterium bifidum* NCIMB 41171. *International Dairy Journal* **17**, 648–656.

Guan, J., Yang, F.Q. & Li, S.P. (2010) Evaluation of carbohydrates in natural and cultured cordyceps by pressurized liquid extraction and gas chromatography coupled with mass spectrometry. *Molecules* **15**, 4227-4241.

Guillaumie, F., Justesen, S.F.L., Mutenda, K.E., Roepstorff, P., Jensen, K.J. & Thomas, O.R.T. (2006) Fractionation, solid-phase immobilization and chemical degradation of long pectin oligogalacturonides. Initial steps towards sequencing of oligosaccharides. *Carbohydrate Research* **341**, 118-129.

Gullón, P., Moura, P., Esteves, M.P., Gtrio, F.M., Dosearleminguez, H. & Parajo, J.C. (2008) Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic bacteria. *Journal of Agricultural and Food Chemistry* **56**, 7482-7487.

Gullón, B., Yanez, R., Alonso, J.L. & Parajo, J.C. (2010) Production of oligosaccharides and sugars from rye straw: A kinetic approach. *Bioresource Technology* **101**, 6676-6684.

Gullón, P., González-Muñoz, M.J. & Parajo, J.C. (2011) Manufacture and prebiotic potential of oligosaccharides derived from industrial solid wastes. *Bioresource Technology* **102**, 6112-6119.

Han, D. & Row, K.HY. (2012) Trends in liquid-phase microextraction, and its application to environmental and biological samples. *Microchimica Acta* **176**: 1-22.

Hernández, O., Ruiz-Matute, A., Olano, A., Moreno, F.J. & Sanz, M.L. (2009) Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. *International Dairy Journal* **19**, 531-536.

Hernández-Hernández, O., Côté, G.L., Kolida, S., Rastall, R.A., Sanz, M.L. (2011) In Vitro Fermentation of Alternansucrase Raffinose-Derived Oligosaccharides by Human Gut Bacteria. *Journal of Agricultural and Food Chemistry* **59**, 10901-10906.

Heuer, C., Kusters, E., Plattner, T. & Seidel-Morgenstern, A. (1998) Design of the simulated moving bed process based on adsorption isotherm measurements using a perturbation method. *Journal of Chromatography A* **827**, 175-191.

Hotchkiss, A.T., Hicks, K.B., Doner, L.W. & Irwin, P.L. (1991) Isolation of oligogalacturonic acids in gram quantities by preparative HPLC. *Carbohydrate Research* **215**, 81-90.

Hotchkiss, A.T., Lecrinier, S.L. & Hicks, K.B. (2001) Isolation of oligogalacturonic acids up to DP 20 by preparative high-performance anion-exchange chromatography and pulsed amperometric detection. *Carbohydrate Research* **334**, 135-140.

Huebner, J., Wehling, R.L. & Hutkins, R.W. (2007) Functional activity of commercial prebiotics. *International Dairy Journal* **17**, 770–775.

Jin, D.F. & West. C.A. (1984) Characteristics of galacturonic acid oligomers as elicitors of casbene synthetase activity in castor bean seedlings. *Plant Physiology* **74**, 989-992.

Kabel, M.A., Schols, H.A. & Voragen, A.G.J. (2002) Complex xylo-oligosaccharides identified from hydrothermally treated Eucalyptus wood and brewery's spent grain. *Carbohydrate Polymers* **50**, 191-200.

Kamada, T., Nakajima, M., Nabetani, H., Saglam, N. & Iwamoto, S. (2002) Availability of membrane technology for purifying and concentrating oligosaccharides. *European Food Research and Technology* **214**, 435–440.

Keisuke, M.; Tamura, N.; Watanabe, T. K. (1988) Method for producing galactooligosaccharide. *European Patent Application* **EP 0 272 095 A2**.

Kim, J.W. & Mazza, G. (2009) Extraction and Separation of Carbohydrates and Phenolic Compounds in Flax Shives with pH-Controlled Pressurized Low Polarity Water. J*ournal of Agricultural and Food Chemistry* **57**, 1805-1813.

Kishihara, S., Horikawa, H., Tamaki, H., Fujii, S., Nakajima, Y. & Nishio, K. (1989) Continuous chromatographic-separation of palatinose (6-O-alpha-D-glucopyranosyl-D-fructose) and trehalulose (1-O-alpha-D-gluco-pyranosyl-D-fructose) using a simulated moving-bed adsorber. *Journal of Chemical Engineering of Japan* **22**, 434-436.

Knox, J.H. & Gilbert, M.T. (1978) Preparation of porous carbon. *UK Patent* **2035282**.

Knutsen, S.H., Sletmoen, M., Kristensen, T., Barbeyron, T., Kloareg, B. & Potin, P. (2001) A rapid method for the separation and analysis of carrageenan oligosaccharides released by iota- and kappa-carrageenase. *Carbohydrate Research* **331**, 101-106.

Koizumi, K. (2002) HPLC of carbohydrates on graphitized carbon columns In: *Carbohydrate analysis by modern chromatography and electrophoresis* (Ed. Z. El Rassi). pp 103-118 Elsevier, Amsterdam.

Kuhn, R.C & Filho, F. (2010) Purification of fructooligosaccharides in an activated charcoal fixed bed column. *New Biotechnology* **27**, 862-869.

Kuhn, R.C., Palacio, L., Pradanos, P., Hernández, A. & Maugeri, F. (2011) Selection of membranes for purification of fructooligosaccharides. *Desalination and Water Treatment* **27**, 18-24.

Lama-Muñoz, A., Rodriguez-Gutierrez, G., Rubio-Senent, F. & Fernandez-Bolanos, J. (2012) Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids* **28**, 92-104.

Li, W.Y., Li, J.D., Chen, T.Q. & Chen, C.X. (2004) Study on nanofiltration for purifying fructo-oligosaccharides I. Operation modes. *Journal of Membrane Science* **245**, 123-129.

Li, W.Y., Li, J.D., Chen, T.Q., Zhao, Z.P. & Chen, C.X. (2005) Study on nanofiltration for purifying fructo-oligosaccharides II. Extended pore model. *Journal of Membrane Science* **258**, 8-15.

Li, Z., Xiao, M., Lu, L. & Li, Y. (2008) Production of non-monosaccharide and high-purity galactooligosaccharides by immobilized enzyme catalysis and fermentation with immobilized yeast cells. *Process Biochemistry* **43**, 896–899.

Liu, Y.K., Luh, B.S. (1978) Preparation and thin-layer chromatography of oligogalacturonic acids. *Journal of Chromatography* **151**, 39-49.

Lundqvist, J., Teleman, A., Junel, L., Zacchi, G., Dahlman, O., Tjerneld, F. & Stalbrand, H. (2002) Isolation and characterization of galactoglucomannan from spruce (*Picea abies*). *Carbohydrate Polymers* **48**, 29-39.

Lundqvist, J., Jacobs, A., Palm, M., Zacchi, G., Dahlman, O. & Stalbrand, H. (2003) Characterization of galactoglucomannan extracted from spruce (Picea abies) by heat-fractionation at different conditions. *Carbohydrate Polymers* **51**, 4743-4748.

Maischberger, T., Nguyen, T-H., Sukyai, P., Kittl, R., Riva, S., Ludwig, R. & Haltrich, D. (2008) Production of lactose-free galacto-oligosaccharide mixtures: comparison of

two cellobiose dehydrogenases for the selective oxidation of lactose to lactobionic acid. *Carbohydrate Research* **343**, 2140-2147.

Martinez-Ferez, A., Guadix, A. & Guadix, E.M. (2006a) Recovery of caprine milk oligosaccharides with ceramic membranes. *Journal of Membrane Science* **276**, 23-30.

Martinez-Ferez, A., Rudloff, S., Guadix, A., Henkel, C.A., Pohlentz, G., Boza, J.J., Guadix, E.M. & Kunz, C. (2006b) Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *International Dairy Journal* **16**, 173-181.

Martinez-Ferez, A., Zapata, J.E., Guadix, A., Almecija, M.C., Gomez, M., Guadix, E.M. (2009) Obtention of goat milk permeates enriched in lactose-derived oligosaccharides. *Desalination* **245**, 730-736.

Matella, N.J., Dolan, K.D. & Lee, Y.S. (2006) Comparison of galactooligosaccharide production in free-enzyme ultrafiltration and in immobilized-enzyme systems. *Journal of Food Science* **71**, C363-C368.

Matsubara, Y., Iwasaki, K., Nakajima, M., Nabetani, H. & Nakao, S. (1996) Recovery of oligosaccharides from steamed soybean waste water in tofu processing by reverse osmosis and nanofiltration membranes. *Bioscience Biotechnology and Biochemistry* **60**, 421-428.

Melotto E., Greve L.C. & Labavitch J.M. (1994) Cell wall metabolism in ripening fruit (VII. biologically active pectin oligomers in ripening tomato (*Lycopersicon esculentum* Mill.) Fruits). *Plant Physiology* **106**, 575-581.

Mihlbachler, K. & Dapremont, O. (2005) Simulated Moving Bed Chromatography offers real attractions. *Chemical Processing* **68**, 38-41.

Moerman, F.T., Van Leeuwen, M.B. & Delcour, J.A. (2004) Enrichment of higher molecular weight fractions in inulin. J*ournal of Agricultural and Food Chemistry* **52**, 3780-3783.

Montañés, F., Fornari, T., Martin-Alvarez, P.J., Corzo, N., Olano, A. & Ibanez, E. (2006) Selective recovery of tagatose from mixtures with galactose by direct extraction with supercritical CO2 and different cosolvents. *Journal of Agricultural and Food Chemistry* **54**, 8340-8345.

Montañés, F., Olano, A., Ibáñez, E. & Fornani, T. (2007) Modelling solubilities of sugars in alcohols based on original experimental data. *Thermodynamics* **53**, 2411-2418.

Montañés, F., Corzo, N., Olano, A., Reglero, G., Ibanez, E. & Fornari, T. (2008) Selective fractionation of carbohydrate complex mixtures by supercritical extraction with CO_2 and different co-solvents. *Journal of Supercritical Fluids* **45**, 189-194.

Montañés, F., Olano, A., Reglero, G., Ibanez, E. & Fornari, T. (2009) Supercritical technology as an alternative to fractionate prebiotic galactooligosaccharides. *Separation and Purification Technology* **66**, 383-389.

Montañés, F. Fornani, T., Olano, A., Ibañez, E. (2010) Supercritical fluid purification of complex carbohydrate mixtures produced by enzimatic transglycosilation and isomerized with complexating reagents. *Journal of Supercritical Fluids* **53**, 25–33

Montañés, F., Fornari, T., Olano, A. & Ibanez, E. (2012) Isolation of prebiotic carbohydrates by supercritical fluid extraction. Scaling-up and economical feasibility. J*ournal of Chromatography A* **1250**, 92-98.

Montilla, A., van de Lagemaat, J., Olano, A. & del Castillo, MD. (2006) Determination of oligosaccharides by conventional high-resolution gas chromatography. C*hromatographia* **63**, 453-458.

Nabarlatz, D., Torras, C., Garcia-Valls, R. & Montane, D. (2007) Purification of xylo-oligosaccharides from almond shells by ultrafiltration. S*eparation and Purification Technology* **53**, 235-243.

Nagel, C.W. & Wilson, T.M. (1969) The isolation of oligogalacturonic acids by column chromatography. *Journal of Chromatography* **41**, 410-416.

Nicoud, R. M. (1998) Simulated Moving Bed (SMB): some possible applications for biotechnology. In: *Bioseparation and Bioprocessing: Biochromatography, Membrane Separations, Modeling, Validation* (ed. G. Subramanian). Wiley-VCH, Weinheim pp 1-39.

Nishizawa, K., Nakajima, M. & Nabetani, H. (2000) A forced-flow membrane reactor for transfructosylation using ceramic membrane. *Biotechnology and Bioengineering* **68**, 92-97.

Nobre, C., Teixeira, J.A., Rodrigues, L., Severino, A., Retamal, C., De Weireld, G. & Van de Wouwer, A. (2010) Operating conditions of a simulated moving bed chromatography unit for the purification of fructo-oligosaccharides. In: *6th international conference on simulation and modelling in the food and bio-industry (FOODSIM 2010)* (eds. V. Cadavez & D. Thiel). Eurosis, Ghent pp 216-218.

Olano, A. (1979) Solubility of lactose and lactulose in alcohols. *Journal of Food Science and Technology* **16**, 260-261.

Olano-Martin, E., Mountzouris, K.C., Gibson, G.R. & Rastall, R.A. (2001) Continuous production of pectic oligosaccharides in an enzyme membrane reactor. *Journal of Food Science* **66**, 966-971.

Osawa, T. & Tsuji, T. (1987) Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Annual Reviews on Biochemistry* **56**: 21-42.

Passos, Claudia P.; Neves, Jose M. G. V.; Coimbra, Manuel A. (2011)Microwave-assisted extraction of arabinogalactans from coffee residue - study of process conditions In *Proceedings of the 7th international conference on polysaccharides-glycoscience* (Eds. Rapkova, R; Copikova, J; Sarka, E) pp. 156-158 Prague, Czech Republic.

Petzelbauer, I., Splechtna, B. & Nidetzky, B. (2002) Development of an ultrahigh-temperature process for the enzymatic hydrolysis of lactose. III. utilization of two thermostable beta-glycosidases in a continuous ultrafiltration membrane reactor and galacto-oligosaccharide formation under steady-state conditions. *Biotechnology and Bioengineering* **77**, 394-404.

Pinelo, M., Jonsson, G. & Meyer, A.S. (2009) Membrane technology for purification of enzymatically produced oligosaccharides: Molecular and operational features affecting performance. S*eparation and Purification Technology* **70**, 1-11.

Pocedicova, K., Germain, B., Krausova, J. & Curda, L. (2010) Preparation of galactooligosaccharides using crude cell extract from lactic acid bacteria. *Journal of Biotechnology* **150**, S329-S329.

Pronyk, C. & Mazza, G. (2011) Optimization of processing conditions for the fractionation of triticale straw using pressurized low polarity water. *Bioresource Technology* **102**, 2016-2025.

Pronyk, C. & Mazza, G. (2012) Fractionation of triticale, wheat, barley, oats, canola, and mustard straws for the production of carbohydrates and lignins. *Bioresource Technology* **106**, 117-124.

Rice, G., Barber, A., O'Connor, A., Stevens, G. &; Kentish, S. (2009) Fouling of NF membranes by dairy ultrafiltration permeates. *Journal of Membrane Science* **330**, 117-126.

Rivas, S., Gullón, B., Gullón, P., Alonso, J.L & Parajo, J.C. (2012) Manufacture and properties of bifidogenic saccharides derived from wood mannan. *Journal of Agricultural and Food Chemistry* **60**, 4296-4305.

Rodriguez-Sanchez, S., Ruiz-Matute, A.I., Alanon, M.E., Perez-Coello, M.S., Julio-Torres, L.F., Morales, R. & Martinez-Castro, I. (2010) Analysis of cyclitols in different Quercus species by gas chromatography-mass spectrometry. *Journal of the Science of Food and Agriculture* **90**, 1735-1738.

Ruiz-Aceituno, L., Ramos, L., Martinez-Castro, I. & Sanz, M.L. (2012) Low Molecular Weight Carbohydrates in Pine Nuts from *Pinus pinea* L. *Journal of Agricultural and Food Chemistry* **60**, 4957-4959.

Ruiz-Aceituno, L., Sanz, M.L. & Ramos, L. (2013) Use of ionic liquids in analytical sample preparation of organic compounds of food and environmental samples. *TrAC Trends in Analytical Chemistry* In press. doi:http://dx.doi.org/10.1016/j.trac.2012.12.006

Ruiz-Matute, A.I., Sanz, M.L., Corzo, N., Martin-Alvarez, P.J., Ibanez, E., Martinez-Castro, I. & Olano, A. (2007) Purification of lactulose from mixtures with lactose using pressurized liquid extraction with ethanol-water at different temperatures. *Journal of Agricultural and Food Chemistry* **55**, 3346-3350.

Ruiz-Matute, A.I., Ramos, L., Martinez-Castro, I. & Sanz, M.L. (2008) Fractionation of honey carbohydrates using pressurized liquid extraction with activated charcoal. *Journal of Agricultural and Food Chemistry* **56**, 8309-8313.

Ruiz-Matute, A.I., Brokl, M., Sanz, M.L., Soria, A.C., Cote, G.L., Collins, M.E. & Rastall, R.A. (2011) Effect of Dextransucrase Cellobiose Acceptor Products on the Growth of Human Gut Bacteria. J*ournal of Agricultural and Food Chemistry* **59**, 3693-3700.

Sanz, M.L., Cote, G.L., Gibson G.R. & Rastall, R. (2005) Prebiotic Properties of Alternansucrase Maltose-Acceptor Oligosaccharides. *Journal of Agricultural and Food Chemistry* **53**, 5911-5916.

Sanz, M.L., Cote, G.L., Gibson, G.R. & Rastall, R.A. (2006) Selective fermentation of gentiobiose-derived oligosaccharides by human gut bacteria and influence of molecular weight. *FEMS Microbiology Ecology* **56,** 383-388.

Sanz, M. L. & Martinez-Castro, I. (2007) Recent developments in sample preparation for chromatographic analysis of carbohydrates. *Journal of Chromatography A* **1153**, 74-89.

Schols, H.A., Voragen, A.G. J. & Colquhoun, I. J. (1994) Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. *Carbohydrate Research* **256**, 97–111.

Schulte, M. & Strube, J. (2001) Preparative enantioseparation by simulated moving bed chromatography. *Journal of Chromatography A* **906**, 399-416.

Searle, L.E.J., Cooley, W.A., Jones, G., Nunez, A., Crudgington, B., Weyer, U., Dugdale, A.H., Tzortzis, G., Collins, J.W., Woodward, M.J. & La Ragione, R.M. (2010) Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and *in vivo*. *Journal of Medical Microbiology* **59**, 1428–1439.

Sen, D., Sarkar, A., Das, S., Chowdhury, R. & Bhattacharjee, C. (2012) Batch Hydrolysis and Rotating Disk Membrane Bioreactor for the Production of Galacto-oligosaccharides: A Comparative Study. *Industrial & Engineering Chemistry Research* **51**, 10671-10681.

Shoaf, K., Mulvey, G.L., Armstrong, G.D. & Hutkins, R.W. (2006) Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infection and Immunity* **74**, 6920–6928.

Sinclair, H.R., de Slegte, J., Gibson, G.R. & Rastall, R.A. (2009) Galactooligosaccharides (GOS) Inhibit Vibrio cholerae Toxin Binding to Its GM1 Receptor. *Journal of Agricultural and Food Chemistry* **57**, 3113-3119.

Smith, D.F., Zopf, D.A. & Ginsburg, V. (1978) Fractionation of sialyl oligosaccharides of human milk by ion-exchange chromatography. *Analytical Biochemistry* **85**, 602-608.

Song, T., Pranovich, A. & Holmbom, B. (2012) Hot-water extraction of ground spruce wood of different particle size. *Bioresources* **7**, 4214-4225.

Spiro, M.D., Kates, K.A., Koller, A.L., Oneill, M.A., Albersheim, P. & Darvill, A.G. (1993) Purification and characterization of biologically-active 1,4-linked alpha-deuterium-oligogalacturonides after partial digestion of polygalacturonic acid with endopolygalacturonase. *Carbohydrate Research* **247**, 9-20.

Splechtna, B.,Petzelbauer, I., Baminger, U., Haltrich, D., Kulbe, K.D. & Nidetzky, B. (2001) Production of a lactose-free galacto-oligosaccharide mixture by using selective enzymatic oxidation of lactose into lactobionic acid. *Enzyme and Microbial Technology* **29**, 6-7.

Strum, J.S., Aldredge, D., Barile, D. & Lebrilla, C.B. (2012) Coupling flash liquid chromatography with mass spectrometry for enrichment and isolation of milk oligosaccharides for functional studies. *Analytical Biochemistry* **424**, 87–96.

Sun, H.J., Yoshida, S., Park, N.H. & Kusakabe, I. (2002) Preparation of (1→4)-β-d-xylooligosaccharides from an acid hydrolysate of cotton-seed xylan: suitability of cotton-seed xylan as a starting material for the preparation of (1→4)-β-d-xylooligosaccharides. *Carbohydrate Research* **337**, 657-661.

Suzuki, T., Tomita-Yokotani, K., Yoshida, S., Takase, Y., Kusakabe, I & Hasegawa, K. (2002) Preparation and isolation of oligogalacturonic acids and their biological effects in cockscomb (*Celosia argentea* L.) seedlings. *Journal of Plant Growth Regulation* **21**, 209-215.

Swennen, K., Courtin, C.M., Van der Bruggen, B., Vandecasteele, C. & Delcour, J.A. (2005) Ultrafiltration and ethanol precipitation for isolation of arabinoxylooligosaccharides with different structures. *Carbohydrate Polymers* **62**, 283-292.

Tsuru, T., Izumi, S., Yoshioka, T. & Asaeda, M. (2000) Temperature effect on transport performance by inorganic nanofiltration membrane. *AIChE Journal* **46**, 565-574.

Vanneste, J., De Ron, S., Vandecruys, S., Soare, S.A., Darvishmanesh, S. & Van der Bruggen, B. (2011) Techno-economic evaluation of membrane cascades relative to simulated moving bed chromatography for the purification of mono- and oligosaccharides. *Separation and Purification Technology* **80**, 600-609.

Vegas, R., Luque, S., Alvarez, J.R., Alonso, J.L., Dominguez, H. & Parajo, J.C. (2006) Membrane-assisted processing of xylooligosaccharide-containing liquors. *Journal of Agricultural and Food Chemistry* **54**, 5430-5436.

Vegas, R., Moure, A., Dominguez, H., Parajo, J.C., Alvarez, J.R. & Luque, S. (2008) Evaluation of ultra- and nanofiltration for refining soluble products from rice husk xylan. *Bioresource Technology* **99**, 5341-5351.

Vente, J.A., Bosch, H., de Haan, A.B., & Bussmann, P.J.T. (2005) Comparison of sorption isotherms of mono- and disaccharides relevant to oligosaccharide separations for Na, K, and Ca loaded cation exchange resins. C*hemical Engineering Communications* **192**, 23-33.

Wang, F., Hu, G.H., Xiao, J.B. & Liu, Y. (2011) Improvement in the productivity of xylooligosaccharides from rice straw by feed xylanase with ultrafiltration. A*rchives of Biological Sciences* **63**, 161-166.

Welton, T. (1999) Room-temperature ionic liquids. Solvents for synthesis and catalysis. *Chemical Reviews* **99**, 2071-2083.

Whistler, R.L. & Durso, D.F. (1950) Chromatographic separation of sugars on charcoal. *Journal of American Chemical Society* **72**, 677-679.

Yoon, S.H., Mukerjea, R. & Robyt, J.F. (2003) Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. *Carbohydrate Research* **338**, 1127–1132.

Yoshida, T., Tsubaki, S., Teramoto, Y. & Azum, J. (2010) Optimization of microwave-assisted extraction of carbohydrates from industrial waste of corn starch production using response surface methodology. *Bioresource Technology* **101**, 7820–7826.

Yuan, Q.P., Zhang, H., Qian, Z.M. & Yang, X.J. (2004) Pilot-plant production of xylo-oligosaccharides from corncob by steaming, enzymatic hydrolysis and nanofiltration. *Journal of Chemical Technology and Biotechnology* **79**, 1073-1079.

Zakrzewska, M.E., Bogel-Uukasik, E. & Bogel-Yukasik, R. (2010) Solubility of carbohydrates in ionic liquids. *Energy Fuels* **24**, 737–745.

Zhan, D., Janssen, P. & Mort, A.J. (1998) Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. *Carbohydrate Research* **308**, 373–380.

Zhang, L., Su, Y.L., Zheng, Y., Jiang, Z.Y., Shi, J.F., Zhu, Y.Y. & Jiang, Y.J. (2010) Sandwich-structured enzyme membrane reactor for efficient conversion of maltose into isomaltooligosaccharides. *Bioresource Technology* **101**, 9144-9149.sen

Zhang, Z., Yang, R.J., Zhang, S., Zhao, H.F. & Hua, X. (2011) Purification of lactulose syrup by using nanofiltration in a diafiltration mode. *Journal of Food Engineering* **105**, 112-118.

Zhang, M., Weina, D. & Hua, B. (2012) Physicochemical Characterization of a Low-Molecular-Weight Fructooligosaccharide from Chinese Cangshan Garlic (*Allium sativum* L.). *Journal of Agricultural and Food Chemistry* **60**, 9462-9467.

Zhao, H.F., Hua, X., Yang, R.J., Zhao, L.M., Zhao, W. & Zhang, Z. (2012) Diafiltration process on xylo-oligosaccharides syrup using nanofiltration and its modeling. *International Journal of Food Science and Technology* **47**, 32-39.

Table 13.1. Some applications of membrane techniques for the concentration and purification of food bioactive oligosaccharides.

Target oligosaccharides	Starting substrates	Membrane technique	Membrane system	Operating conditions				Reference
				Effective area (cm^2)	T (°C)	P (bar)[a]	Permeate flux (L/h per m^2)[b]	
Lactulose	Lactulose syrup produced by the alkali isomerization of lactose	NF	Thin-film composite spiral-wound NF membrane module (NF2A-2540 MIL	12,000	30	6-26	10-120	Zhang *et al*. (2011)
GOS	Enzymatic synthesis from lactose buffered solutions (continuous process)	UF	Laboratory-scale reactor system with a regenerated cellulose membrane (NMWCO 10 kDa)	50	45	0.75-2.75	27.75-47.35	Chockchaisawasdee *et al*. (2005)
GOS	Commercial mixture of GOS containing digestible mono- and disaccharides	NF	Stirred cell equipped with a flat sheet cellulose acetate membrane, NF-CA-50 (50% rejection of NaCl), and a thin film trilaminate polyethersulphone	40	20-25	40	30-80	Goulas *et al*. (2003)

			membrane, NF-TFC-50 (50% rejection of NaCl)					
GOS	Enzymatic synthesis from whey	Combined UF, NF and RO	Polyethersulfone UF membranes (NMWCO 50 and 5 kDa), polyamide thin-film composite NF membrane with a microporous polysulfone supporting layer (9.8% rejection of NaCl), polypropylene thin-film composite RO membrane with a porous support (≥97% rejection of NaCl)	200 (UF) 25 (NF)	25	1.5 (UF) 10 (NF) 20 (RO)		Das *et al*. (2011)
FOS (DPs ≥4)	Commercial mixture of FOS containing digestible mono- and disaccharides	UF	Stirred UF cell with an YM 1 regenerated cellulose membrane with a NMWCO of 1 kDa.		4	3.0		Montilla *et al*. (2006)
FOS	FOS containing digestible mono- and disaccharides	NF	Forced-flow membrane reactor using five different tubular ceramic membranes of symmetric and asymmetric types	55.6	50	3.5	10-30[c]	Nishizawa *et al*. (2000)

FOS	Commercial powdered chicory rootstock	Combined UF and NF	A GR61PP UF membrane made of polysulfone (NMWCO 20 kDa) and a G10 NF membrane made of polyamide composite (30% rejection of NaCl)	3,600	30 (UF) 25 (NF)	3.5 (UF) 4.0 (NF)	17-118 (UF) 11.2-82.0 (NF)	Kamada *et al.* (2002)
XOS	Liquors from almond shells autohydrolysis	UF	Bench-scale plant equipped with UF thin-film polymeric , flat sheet of polymeric membranes with a NMWCO of 1, 2.5, 3.5 and 8 kDa	12.57	25	2.6-9.0	1.1-55	Nabarlatz *et al.* (2007)
XOS	Liquors from rice husk autohydrolysis	NF	Small-scale pilot unit using a monolithic ceramic membrane made of TiO_2/ZrO_2 (95% rejection of monosaccharides)	2,500	26	6-14	10-60	Vegas *et al.* (2006) Vegas *et al.* (2008)
XOS	Liquors from rice husk autohydrolysis	Combined UF and NF	Tubular ceramic membranes with NMWCO of 1 and 15 kDa.	220		8.0 (UF) 10.0 (NF) 4.0 (UF)		Gullón *et al.* (2010)
AXOS	Enzymatically produced arabinoxylan	UF	Stirred cell with UF membranes with NMWCO of 5, 10 and 30		Room temperature	4.0		Swennen *et al.* (2005)

	hydrolysates from wheat flour		kDa					
Pectic oligosaccharides	High methylated citrus pectin and low methylated apple pectin	UF	Stirred cells with an Amicon PM 10 UF membrane with a NMWCO of 10 kDa	28.7	30			Olano-Martin *et al*. (2001)
Hemicellulose-derived oligosaccharides	Liquors from *Pinus pinaster* wood autohydrolysis	UF	Stirred cell with an UF regenerated cellulose membranes with NMWCO of 1 kDa	41.8		4.0		González-Muñoz *et al*. (2011)
IMOs	Enzymatic synthesis from maltose buffered solutions	UF	Sandwich-structured enzyme membrane reactor with the enzyme immobilized between two sheets of UF membranes (PES/Pluronic F127 and PES/PEG	287		1.0	26.53-209.06	Zhang *et al*. (2010)
Lactose-derived oligosaccharides	Goat Milk	Combined UF and NF	Two-stage tangential filtration process using multichannel tubular ceramic membranes made	94	30	0.9 (UF) 3.75 (NF)	35.1-73.4 (UF) 18.8-28.4 (NF)	Martinez Ferez *et al*. (2006a) Martinez Ferez

			of ZrO_2-TiO_2 (NMWCO 50 kDa for UF and 1 kDa for NF)					*et al*. (2006b)
Soybean-derived oligosaccharides	Steamed soybean waste water from tofu processing	UF and either RO or NF	A polysulfone UF membrane (NMWCO 20 kDa), a spiral-type RO membrane NTR-7199 (99.3% rejection of NaCl) and an NF membrane NTR-7250 (60% rejection of NaCl)	4,000 (UF) 17,000 (RO and NF)	50 (UF) 25 (NF)	2.0 (UF) 10-50 (RO) 5-30 (NF)	2.5-15 x 10^{6d}	Matsubara *et al*. (1996)

[a] Trans-membrane differential pressure

[b] Dependent on specific pressure applied in each work.

[c] cm s^{-1} per m^2

[d] m^3 s^{-1} per m^2

Table 13.2. Comparison of fractionation techniques to obtain food bioactive oligosaccharides based on their molecular weight or glycosidic linkages and/or monosaccharide composition.

	Fractionation based on	
Techniques / Treatments	Molecular weight	Glycosidic linkages and/or monomer composition
Activated charcoal	XXX	-
Size exclusion chromatography	XXX	-
Ion exchange chromatography	XX	XX
Membrane-based	XX	-
Yeast	X	XX
Supercritical Fluid Extraction[a]	X	XX
Pressurized Liquid Extraction[a]	X	XX

XXX= Efficient fractionation.

XX= Moderate fractionation

X= Limited fractionation

- = No fractionation

[a] = Scarce data